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The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlins and chaplins

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Summary

Streptomycetes form hydrophobic aerial hyphae that eventually septate into hydrophobic spores. Both aerial hyphae and spores possess a typical surface layer called the rodlet layer. We present here evidence that rodlet formation is conserved in the streptomycetes. The formation of the rodlet layer is the result of the interplay between rodlins and chaplins. A strain of *Streptomyces coelicolor* in which the rodlin genes *rdlA* and/or *rdlB* were deleted no longer formed the rodlet layer. Instead, these surfaces were decorated with fine fibrils. Deletion of all eight chaplin genes (strain Δ*chpABCDEFGH*) resulted in the absence of the rodlet layer as well as the fibrils at surfaces of aerial hyphae and spores. Apart from coating these surfaces, chaplins are involved in the escape of hyphae into the air, as was shown by the strong reduction in the number of aerial hyphae in the Δ*chpABCDEFGH* strain. The decrease in the number of aerial hyphae correlated with a lower expression of the *rdl* genes in the colony. Yet, expression per aerial hypha was similar to that in the wild-type strain, indicating that expression of the *rdl* genes is initiated after the hypha has sensed that it has grown into the air.

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Introduction

Streptomycetes exhibit a complex life cycle. These Gram-positive soil bacteria form a colonizing mycelium within the moist substrate by multinucleoid hyphae that grow at their apices. After a feeding substrate mycelium has been established, hyphae leave the aqueous environment to grow into the air. These aerial hyphae differentiate by forming chains of uninucleoid cells, which metamorphose into pigmented spores. These spores are dispersed and may give rise to a new mycelium. Growth into the air is accompanied by a change in the surface properties. Surfaces of hyphae in the moist substrate are hydrophilic, whereas those of aerial hyphae and spores are hydrophobic. Hydrophobicity is attributed to several surface layers, one of which is the rodlet layer. This layer consists of a mosaic of 8- to 10-nm-wide rods (Wildermuth *et al*., 1971; Smucker and Pfister, 1978). Formation of aerial hyphae and spores has been best studied in *Streptomyces coelicolor* (for recent reviews, see Chater, 1998; 2001; Kelemen and Buttner, 1998; Wösten and Willey, 2000). Three types of secreted proteins, namely SapB (Willey *et al*., 1991; 1993), chaplins (Claessen *et al*., 2003; Elliot *et al*., 2003) and rodlins (Claessen *et al*., 2002), were shown to be involved in the formation of aerial hyphae and spores.

SapB is a small secreted peptide of 18 amino acids that is produced when *S. coelicolor* is grown in rich medium (Willey *et al*., 1991). It lowers the medium surface tension from 72 to 32 mJ m^{-2}, thus enabling hyphae to breach the medium–air interface to grow into the air (Tillotson *et al*., 1998). Strikingly, no *sapB* gene could be identified in the *S. coelicolor* genome sequence (Bentley *et al*., 2002), and it was therefore proposed to be synthesized non-ribosomally. SapB is not expected to aid in the surface hydrophobicity of aerial hyphae and spores as this peptide could not be detected at the surfaces of these structures (Wösten and Willey, 2000). This hypothesis was strengthened by the observation that *S. coelicolor* does form aerial hyphae in minimal medium despite the absence of SapB under these culture conditions.

Chaplins were identified as a class of hydrophobic proteins involved in the formation of aerial hyphae in *S. coelicolor* (Claessen *et al*., 2003; Elliot *et al*., 2003). This class

consists of eight members, ChpA–H, that are inserted into the cell walls of aerial hyphae of cultures grown on rich or minimal medium. Within the cell wall, mature forms of ChpD–H (±55 amino acids) and ChpA–C (±225 amino acids) self-assemble into amyloid-like fibrils (Claessen *et al.*, 2003). ChpA–C contain two domains similar to ChpD–H as well as a cell wall sorting signal. This signal explains why these larger chaplins could not be extracted from cell walls of aerial hyphae. In contrast, the smaller chaplins ChpD–H could be purified from cell walls using trifluoroacetic acid. Assembly of these chaplins at the water–air interface was found to be accompanied by a huge drop in surface tension (from 72 to 26 mJ m^{-2}). This suggested that these proteins could be involved in escape of hyphae from the moist environment into the air. Indeed, ChpE and ChpH were found to be secreted in the culture medium as well as in cell walls of aerial hyphae. The involvement of the chaplins in the formation of these structures was confirmed by gene deletion. Formation of aerial hyphae was strongly affected in a strain in which six *chp* genes were deleted (Δ*chpABCDEH*). The surface of the aerial hyphae produced by the mutant strain still possessed a rodlet layer.

The rodlin proteins RdlA and RdlB were shown to be present at surfaces of aerial hyphae and spores where they form a highly insoluble layer (Claessen *et al.*, 2002). Disruption of both *rdlA* and *rdlB* in *S. coelicolor* (Δ*rdlAB* strains) did not affect the formation and differentiation of aerial hyphae. However, the characteristic rodlet layer was absent. We show here that both rodlin and chaplin proteins are involved in the formation of the rodlet layer of streptomycetes. Our results indicate that the small chaplins, ChpD–H, assemble into fibrils that are aligned into rodlets by the action of the non-redundant RdlA and RdlB rodlins.

Results

The rdlA *and* rdlB *genes are contained on a conserved gene cluster in the genomes of streptomycetes*

Streptomyces coelicolor and *Streptomyces lividans* contain identical copies of the *rdlA* and *rdlB* genes (Claessen *et al.*, 2002). Hybridization of these genes with genomic DNA of various streptomycetes indicated the ubiquitous occurrence of rodlin genes in this genus (Claessen *et al.*, 2002). To isolate the homologues of *Streptomyces tendae* and *Streptomyces griseus*, cosmid libraries were hybridized with probes directed against the coding sequences of *rdlA* and *rdlB*. Both genes hybridized with the overlapping *S. tendae* cosmids C18 and C60 and the *S. griseus* cosmid 12A10 (data not shown). The hybridizing fragments were contained on 3.2, 4.2 and 3.6 kb *Sal*I fragments respectively. These fragments were cloned and sequenced. The overlapping *S. tendae* cosmids C18 and C60 contained two open reading frames (ORFs) representing the *S. tendae* homologues of RdlA and RdlB (Fig. 1). The genes, called *STrdlA* and *STrdlB*, are highly homologous to their equivalents in *S. lividans* and *S. coelicolor* with respect to the deduced amino acid sequence (*Supplementary material*, Fig. S1) as well as their genetic organization (Fig. 1).

They are divergently transcribed from the putative promoter region contained in the 245 bp intergenic region. Similarly, two rodlin genes, called *SGrdlA* and *SGrdlB*, were identified on cosmid 12A10 of *S. griseus* (*Supplementary material*, Fig. S1). In contrast to *S. coelicolor* and *S. tendae*, the rodlin genes of *S. griseus* are not divergently transcribed (Fig. 1). In between the *S. griseus* rodlin genes, an ORF was identified that was highly homologous to ORF2 of *S. tendae* and SCO2717 of *S. coelicolor* encoding the small chaplin ChpD. In addition,

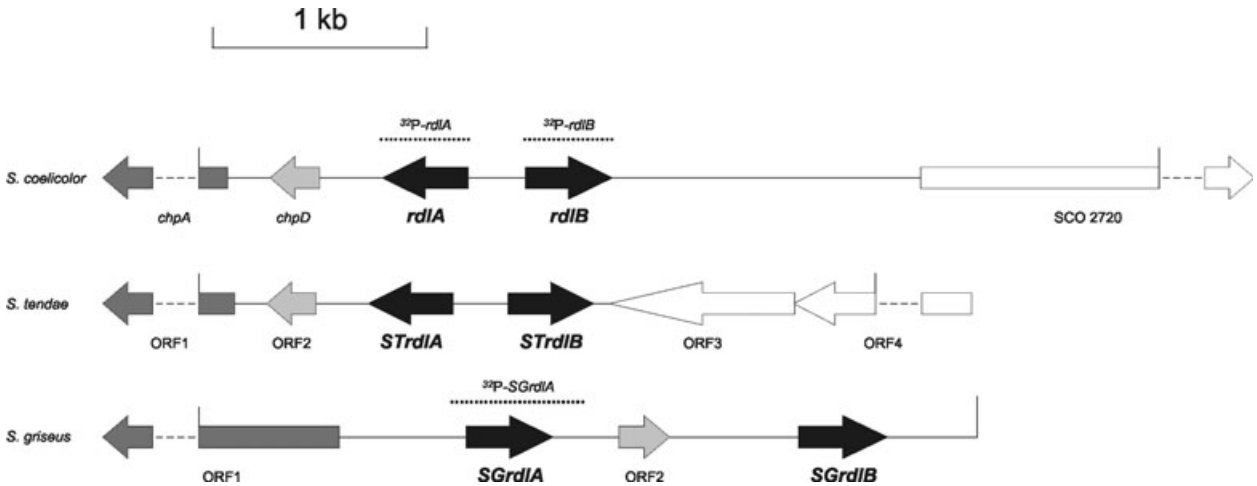

Fig. 1. The genetic organization of *rdl* genes from *S. coelicolor*, *S. tendae* and *S. griseus* is conserved. *rdl* genes are found on part of the genome that also contains *chpD* (indicated in light grey) and *chpA* (indicated in dark grey). Dotted lines indicate probes used in this study.

both *S. griseus* and *S. tendae* clones contained part of an ORF (ORF1) with high similarity to SCO2716 of *S. coelicolor* (Fig. 1) encoding ChpA. These data show that the *rdl* genes are located on a conserved gene cluster in streptomycetes encoding proteins involved in the formation of surface layers.

Expression of rdl *genes correlates with the presence of rodlet-decorated surfaces*

Streptomyces coelicolor, *S. tendae*, *Streptomyces scabies* and *Streptomyces avermitilis* form spores when grown on solid media. In contrast, some *S. griseus* strains also form spores in liquid shaken cultures. The resistance properties of *S. griseus* surface-grown and submerged spores are very similar (Kendrick and Ensign, 1983). Surfaces of spores formed on solid media were analysed by scanning electron microscopy.

In contrast to the *S. coelicolor* Δ*rdlAB* strain (Fig. 2F), rodlets were identified on wild-type spores of *S. coelicolor* (Fig. 2A), *S. tendae* (not shown), *S. scabies* (not shown) and *S. griseus* (Fig. 2B). The abundance and shape of the rodlets in the different strains were indistinguishable. To assess whether rodlets were restricted to aerial spores, we analysed surfaces of *S. griseus* spores formed in liquid cultures by freeze-fracturing. Interestingly, like spores produced by aerial hyphae (Fig. 2C), spores produced by submerged cultures of *S. griseus* were decorated with

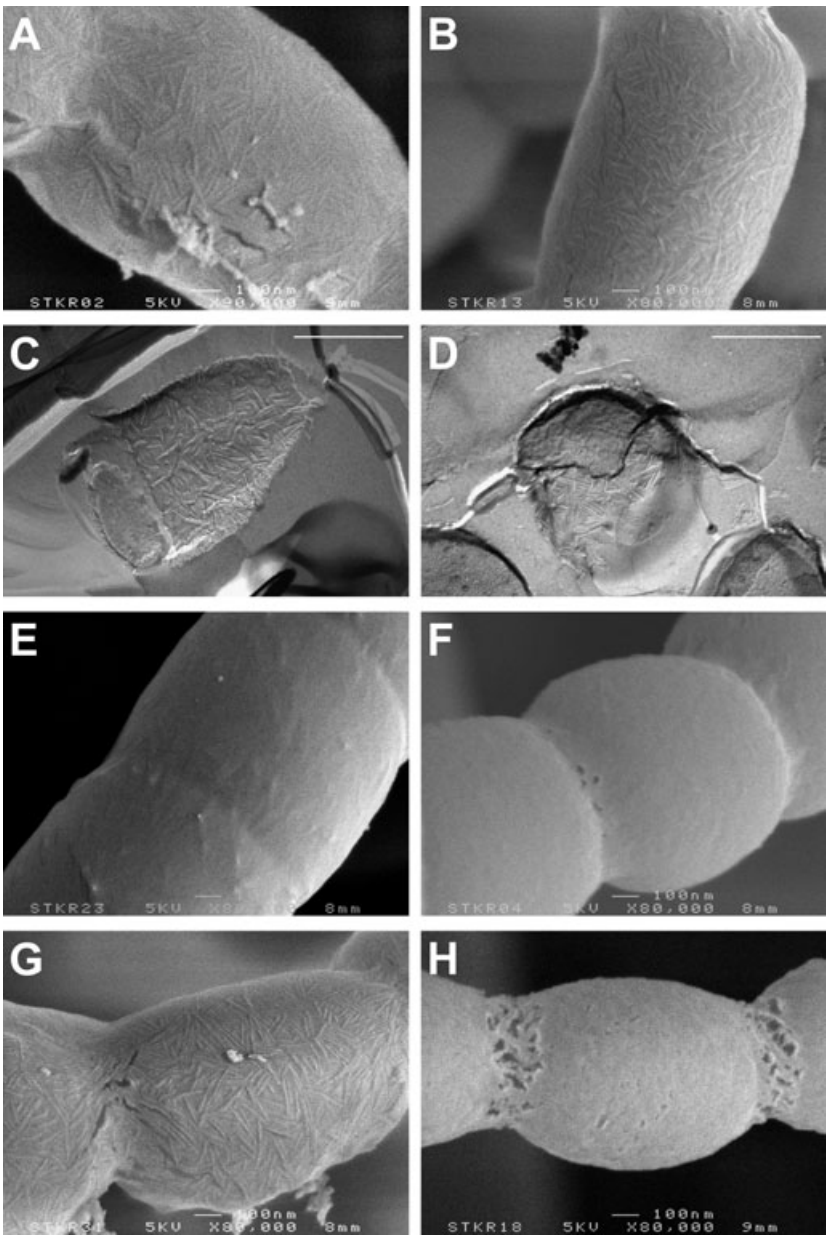

Fig. 2. Spores of *S. coelicolor* (A) and *S. griseus* (B and C) grown on solid media are covered with a rodlet layer. Rodlets are also observed on *S. griseus* spores produced by submerged hyphae (D). These rodlets are very similar to those produced by aerial hyphae (C). On the other hand, no rodlets were observed at surfaces of spores of *S. avermitilis* (E) or the Δ*rdlAB* strain of *S. coelicolor* (F). The latter strain could be complemented by the *rdl* genes from *S. tendae* (G) and *S. griseus* (not shown). Deletion of either *rdlA* (not shown) or *rdlB* (H) is sufficient to eradicate rodlets in *S. coelicolor*. Bars indicate 500 nm (C and D) or 100 nm (A, B, E–H). Surfaces were studied by scanning electron microscopy (A, B, E, F–H) and freeze-fracturing (C and D).

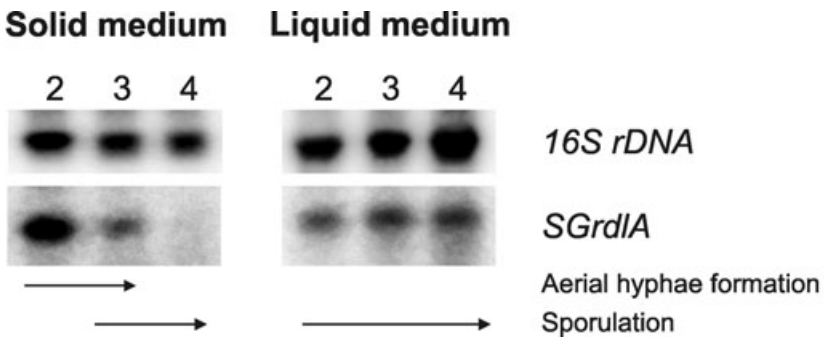

Fig. 3. Temporal expression of *SGrdlA* of *S. griseus* grown on solid medium (left) or as a liquid shaken culture (right) as determined by Northern analysis. Expression of *SGrdlA* coincides with the formation of aerial hyphae on solid medium and with the formation of spores in submerged cultures. Northern blots were rehybridized with 16S rDNA as a loading control and to confirm the integrity of the RNA. Numbers indicate the age of cultures in days.

rodlets (Fig. 2D). This suggested that, in contrast to *S. coelicolor* (Claessen *et al*., 2002), *S. griseus* expresses its rodlin genes not only on solid media but also in liquid shaken cultures (Fig. 3).

Expression of *SGrdlA* in solid cultures decreased after most of the aerial hyphae had been formed. In contrast, expression of *SGrdlA* remained high in liquid shaken medium coinciding with an increase in the number of sporulating hyphae.

Interestingly, *S. avermitilis* did not produce rodlet-decorated spores (Fig. 2E). Accordingly, its genome sequence does not contain rodlin genes (see *Discussion*). These data therefore show that expression of *rdl* genes correlates with the presence of rodlets.

Rodlin genes from S. tendae *and* S. griseus *complement the* S. coelicolor ΔrdlAB *strain*

To analyse whether the *rdl* homologues from *S. tendae* and *S. griseus* functionally complement the *rdlAB* null mutant of *S. coelicolor*, strain Δ*rdlAB6* was transformed with pIJ8630-StC18 or pSET-Sg12A10. In this way, *rdlA* and *rdlB* homologues of *S. tendae* and *S. griseus* were introduced in the λC31 attachment site under the control of their own promoters. Scanning electron microscopy showed that formation of the rodlet layer was restored in both types of transformants. Shape and abundance of the rodlets formed by the rodlins of *S. tendae* (Fig. 2G) and *S. griseus* (not shown) were similar to those observed in the wild-type strain of *S. coelicolor* (Fig. 2A). These data show that rodlins from *S. tendae* and *S. griseus* can functionally complement those of *S. coelicolor*.

RdlA and RdlB are not redundant

Disruption of both *rdlA* and *rdlB* in *S. coelicolor* and *S. lividans* resulted in the absence of rodlets on the surface of aerial hyphae and spores (Claessen *et al*., 2002). To investigate whether these genes are redundant, the entire coding sequence of *rdlA* and/or *rdlB* was replaced by an apramycin resistance cassette in *S. coelicolor* M145 using the polymerase chain reaction (PCR)-targeting disruption system (Gust *et al*., 2003). This resulted in the Δ*rdlA*, Δ*rdlB* and Δ*rdlAB* strains respectively. Formation and differentiation of aerial hyphae in the Δ*rdlA*, Δ*rdlB* and Δ*rdlAB* strains was unaffected on different media and growth conditions (data not shown). However, scanning electron microscopy revealed that, unlike the wild-type strain, rodlets were absent on aerial hyphae and spores of the single (Fig. 2H) and the double knock-out strains (Fig. 2F).

Expression of *rdlB*, however, was affected by the deletion of *rdlA* and vice versa. Northern analysis revealed that the amount of mRNA of *rdlB* was five- to 10-fold lower in the Δ*rdlA* strain (Fig. 4), while a similar reduction was observed for mRNA of *rdlA* in the Δ*rdlB* strain.

In contrast to *rdlB*, two hybridizing mRNAs were observed for *rdlA*. The significance of this is not yet known. To exclude the possibility that the absence of rodlets in the single knock-out strains resulted from the decrease in the expression of the intact *rdl* copy, both single knock-outs were transformed with the integrating plasmids pIJ82-*rdlA* and pIJ82-*rdlB*. Northern analysis revealed that the integration of either plasmid restored the mRNA level of the introduced *rdl* gene (Fig. 4). pIJ82-*rdlA* restored the formation of the rodlet layer in the Δ*rdlA* strain but not in the Δ*rdlB* strain (data not shown). Similarly, formation of rodlets was rescued in the Δ*rdlB* strain by the introduction of pIJ82-*rdlB* (data not shown), but not by pIJ82-*rdlA* (Fig. 4). These data show that RdlA and RdlB are not redundant as they are both necessary for formation of the rodlet layer.

The small chaplins are necessary for fibril formation and assembly of the rodlet layer

Previously, we have shown that rodlets were absent at surfaces of aerial hyphae and spores of the Δ*rdlAB* strain (Claessen *et al*., 2002) but present on those of the Δ*chpABCDEH* strain (Claessen *et al*., 2003). Instead, surfaces of the Δ*rdlAB* strain were decorated with fine fibrils. To establish whether the formation of rodlets *in vivo* also

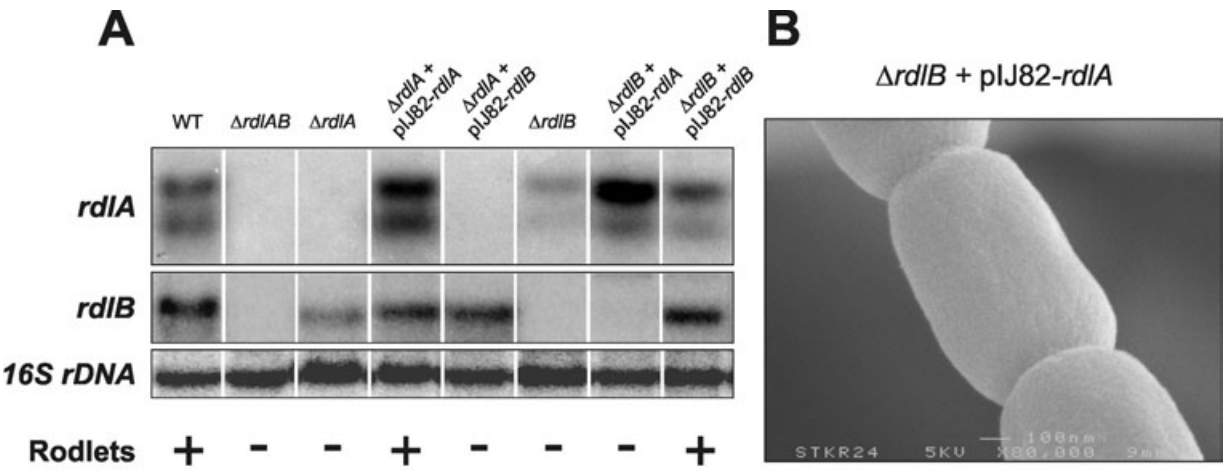

Fig. 4. The *rdlA* and *rdlB* genes are not redundant. RNA isolated from cultures that had started to form aerial hyphae was hybridized with probes directed against *rdlA* or *rdlB* (A). As the deletion of *rdlA* affected the expression of *rdlB* and vice versa, the single knock-out strains were transformed with pIJ82-*rdlA* or pIJ82-*rdlB*. The presence or absence of rodlets is indicated by + or – respectively.
B. The Δ*rdlB* strain transformed with an additional *rdlA* gene does not form rodlets.

depends on the presence of chaplins, the remaining *chpF* and *chpG* genes were deleted in the Δ*chpABCDEH* strain. Formation of aerial hyphae was severely affected in the resulting Δ*chpABCDEFGH* strain (Fig. 5B) compared with that in the wild-type strain (Fig. 5A) and the Δ*chpABCDEH* strain.

The few aerial hyphae formed clumped together and collapsed on top of the submerged hyphae as a result of the binding of water (Fig. 5B). Apparently, these hyphae were hydrophilic. Indeed, when droplets of water were placed on top of sporulating cultures of the Δ*chpABCDEFGH* strain, they spread rapidly, whereas they remained spherical in the wild-type and Δ*chpABCDEH* strains (data not shown). In contrast to the Δ*chpABCDEFGH* strain, the wild-type strain formed abundant aerial hyphae that were hydrophobic and stable for weeks. Surfaces of the few aerial hyphae of the Δ*chpABCDEFGH* strain possessed neither rodlets nor fibrils (Fig. 6A).

Formation of the rodlet layer and surface hydrophobicity were restored in the Δ*chpABCDEFGH* strain after the introduction of C61A, containing *chpF* and *chpG* (not shown). On the other hand, deletion of *chpF* and *chpG* from the wild-type strain did not affect the formation of the hydrophobic rodlet layer at aerial hyphae and spores, showing that ChpF and ChpG are not the only chaplins involved in rodlet formation. From these data, it is concluded that assembly of the rodlet layer is dependent on the presence of both rodlins and the small chaplins, ChpD–H. In addition, we conclude that the formation of

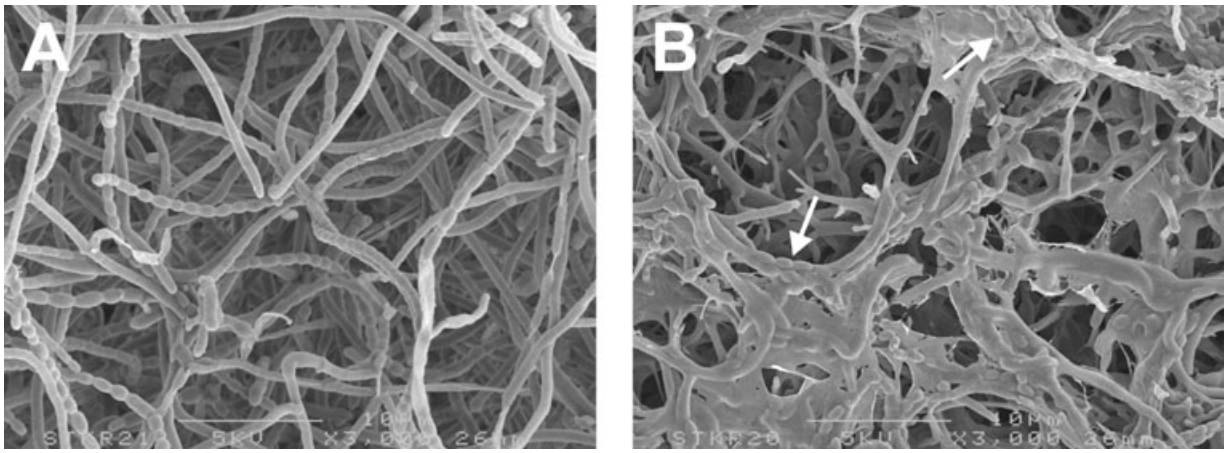

Fig. 5. Aerial growth is strongly affected in the Δ*chpABCDEFGH* strain (B) compared with the wild-type strain (A). The aerial hyphae that are formed by the mutant strain clump together and collapse at the surface of the colony. Arrows indicate spore chains.

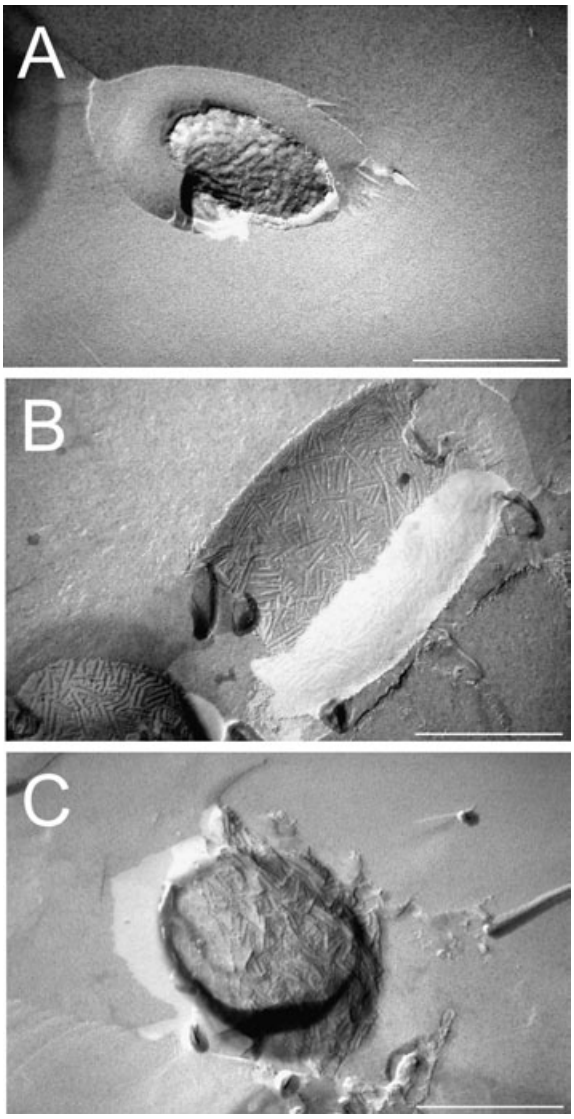

Fig. 6. Freeze-fracturing revealed the absence of rodlets and fibrils at surfaces of the few aerial hyphae in the Δ*chpABCDEFGH* strain (A). In contrast, rodlets were observed at surfaces of spores of the wild-type strain (B) and the Δ*chpABCDEH* strain (C).

the fibrils seen in the Δ*rdlAB* mutant strain depends on chaplin proteins.

Expression of rdlA *and* rdlB *depends on aerial hyphae formation*

The *rdl* genes were identified previously as being specifically expressed in aerial hyphae. To analyse whether expression of *rdlA* and *rdlB* is downregulated in the Δ*chpABCDEH* and Δ*chpABCDEFGH* mutants, total RNA from 3- and 4-day-old cultures grown on solid medium was hybridized with probes directed against *rdlA* or *rdlB*. Compared with the wild-type strain, expression of *rdlA* and *rdlB* was severely affected in the Δ*chpABCDEH* strain, whereas it was even more reduced in the Δ*chpABCDEFGH* strain (Fig. 7A).

To establish whether the reduced expression levels of the *rdl* genes in the *chp* mutant strains resulted from the reduced number of aerial hyphae, the Δ*chpABCDEH* strain was transformed with pIJ8630*a* containing the *eGFP* gene under the control of the *rdlA* promoter. Fluorescence of green fluorescent protein (GFP) was restricted to the few aerial hyphae that had formed (Fig. 7B). Fluorescence per aerial hypha was similar to that of the wild-type strain. We thus conclude that the expression level of *rdlA* is determined by the number of aerial hyphae.

Discussion

Recently, it was shown that not only SapB (Tillotson *et al*., 1998) but also ChpD–H can reduce the water surface tension to allow *S. coelicolor* hyphae to grow into the air (Claessen *et al*., 2003). A strain in which six out of eight chaplins were deleted was severely affected in the formation of aerial hyphae, but the aerial hyphae that had formed still possessed the characteristic rodlet layer. We have shown here that a strain in which all chaplin genes were deleted was further reduced in its ability to form aerial hyphae. Moreover, the few aerial hyphae that had formed now lacked the rodlet layer. Apparently, chaplin proteins play a role in escape of hyphae from the aqueous environment and in coating the aerial hyphae with a rodlet layer. Another class of proteins involved in the formation of this surface layer is the rodlins (Claessen *et al*., 2002). These proteins, however, do not play a role in reduction of the water surface tension as they are only formed by aerial hyphae or hyphae in contact with a hydrophobic solid (Claessen *et al*., 2002).

Deletion of either *rdl* gene was sufficient to prevent rodlet formation, showing that both proteins are essential. The mechanism of rodlet formation seems to be conserved in streptomycetes. *S. tendae* and *S. griseus* also contain two rodlin genes, and these could functionally complement the Δ*rdlAB* strain of *S. coelicolor*. In addition, the genetic organization of the rodlin genes has been conserved in streptomycetes. Homologues of *chpA* and *chpD* were found to be clustered with the rodlin genes within the genomes of these three streptomycetes. These species represent members that are quite diverged. Interestingly, neither aerial hyphae nor spores of *S. avermitilis* were decorated with rodlets. This correlates with the absence of rodlin genes in the genome of this streptomycete (Ikeda *et al*., 2003). In fact, the homologues of *chpA* and *chpD* are also absent while homologues encoding for ChpB, ChpC and the variants detected in the liquid medium (ChpE and ChpH) are

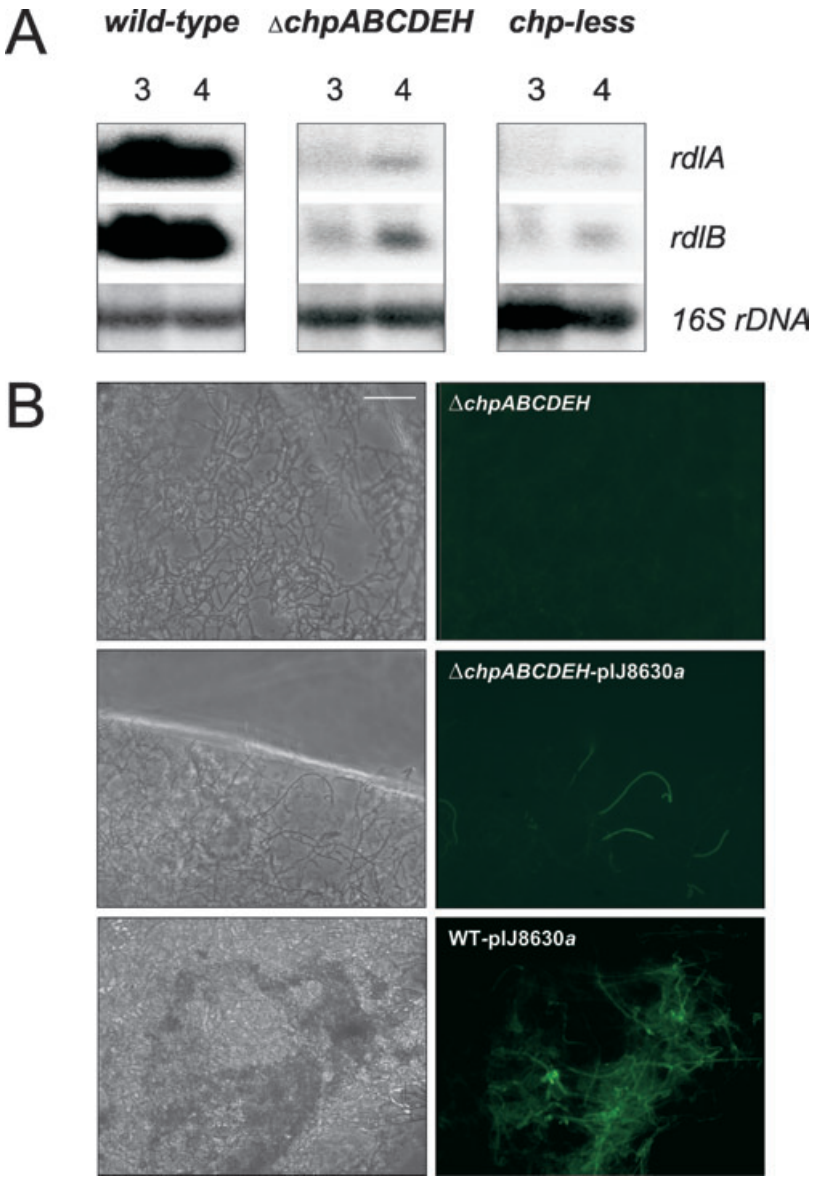

Fig. 7. Accumulation of *rdlA* and *rdlB* mRNA in 3- and 4-day-old cultures of the *S. coelicolor* wild-type strain and the Δ*chpABCDEH* and the chaplin-less Δ*chpABCDEFGH* strains (A). Northern blots were rehybridized with 16S rDNA as a loading control and to confirm the integrity of the RNA. Expression of *rdlA* is restricted to the few aerial hyphae formed in the Δ*chpABCDEH* strain as assessed by GFP fluorescence in the Δ*chpABCDEH*–pIJ8630*a* strain (B). Bar indicates 25 μm.

present (http://avermitilis.ls.kitasato-u.ac.jp/). Apparently, the part of the genome of *S. avermitilis* containing the rodlin gene cluster is lost in evolution without an obvious loss of its ability to differentiate. In agreement, aerial hyphae formation was essentially unaffected in *S. coelicolor* strains lacking four *chp* genes or the *rdl* genes (Claessen *et al*., 2002; 2003).

Formation of rodlets is not restricted to aerial spores *per se*. In contrast to *S. tendae* and *S. coelicolor*, some strains of *S. griseus* form spores in submerged cultures as well. These spores are decorated with rodlets, like the spores produced by aerial hyphae. This indicates that the rodlet layer can be assembled in the absence of a hydrophobic–hydrophilic interface as occurs in filamentous fungi (Wösten *et al*., 1993). Thus, expression seems to determine where rodlets are formed rather than the presence of a hydrophobic–hydrophilic interface.

We showed previously that the rodlet layer was absent at surfaces of spores of the Δ*rdlAB* strain (Claessen *et al*., 2002). Instead, fine fibrils were observed. Interestingly, neither rodlets nor fibrils could be detected at surfaces of the chaplin-less strain Δ*chpABCDEFGH*. Surfaces of the few aerial hyphae that had formed were smooth. A detailed view of the ultrastructure of the rodlet layer suggests that a rodlet consists of two rods that are themselves composed of two smaller fibrils (Wildermuth *et al*., 1971; Claessen *et al*., 2003). These fibrils have a size similar to that of an assembled chaplin fibril (Claessen *et al*., 2003). Based on these data, we propose that the RdlA and RdlB rodlins do not assemble into rodlets

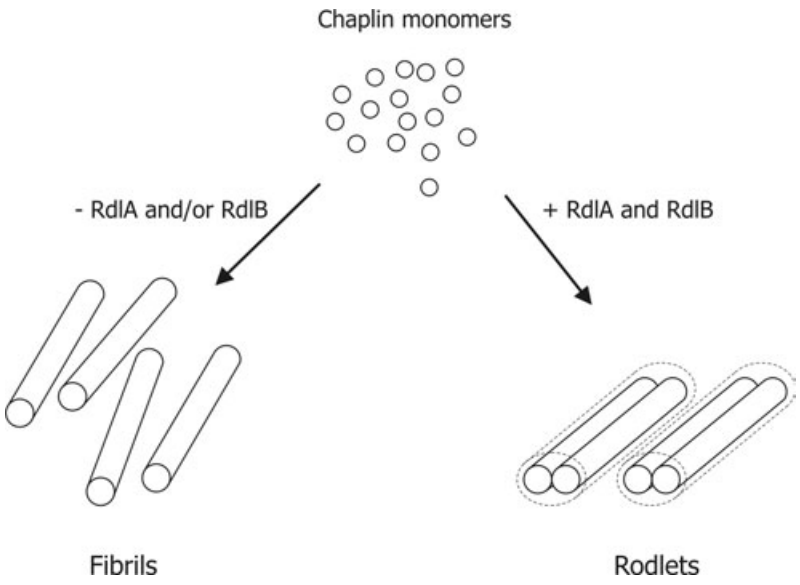

Fig. 8. The formation of rodlets depends on the presence of rodlins and chaplins. Chaplin monomers assemble into small fibrils that are randomly distributed in the absence of rodlins. In the presence of both rodlins RdlA and RdlB, the fibrils are aligned in rodlets. These rodlets consist of two rods, each of which comprises two fibrils.

themselves but align chaplin fibrils into a rodlet layer (Fig. 8).

In the absence of either rodlin, the alignment can no longer take place, and fibrils of small chaplins (ChpD–H) are deposited randomly at the hyphal surface. As rodlets identical to the wild-type strain were observed on surfaces of the Δ*chpABCDEH* strain, ChpF and ChpG are apparently sufficient for the formation of the individual fibrils. However, they can be substituted by the other chaplins because a strain in which the *chpF* and *chpG* genes were deleted still formed rodlets.

We have tried to support the presented model with biochemical data. Rodlins can only be isolated from cell walls using trifluoroacetic acid. However, this solvent completely unfolds the proteins (D. Claessen, unpublished). Similarly, rodlins produced in *Escherichia coli* were also unstructured. We therefore believe that *in vivo* as yet unidentified chaperones are involved in the folding of these proteins.

A 'sensing' mechanism that directs expression of genes encoding proteins involved in aerial hyphae formation

Previously, genes have been isolated that are blocked in the formation of aerial hyphae by interfering in regulatory pathways (Kelemen and Buttner, 1998; Chater, 2001). For instance, *bldN* encodes a developmental sigma factor (Bibb *et al*., 2000). Elliot *et al*. (2003) showed that expression of *rdlA* (and *rdlB*) was severely reduced in a *bldN* mutant, suggesting that expression of this gene is controlled by this or any earlier *bld* gene. If expression of *rdlA* and *rdlB* was solely dependent on the *bld* genes, one would expect these rodlin genes to be similarly expressed in the wild-type and chaplin-less strain. Interestingly, this was not observed. Expression of *rdlA* and *rdlB* in the colony was strongly reduced in the chaplin-less strain Δ*chpABCDEFGH*. However, expression per hypha was unaffected, as was shown using GFP as a reporter. These data strongly suggest that expression of the rodlin genes is initiated when a sensor has signalled that the hypha has left the aqueous environment. The *rdl* genes are expressed not only in hyphae that grow in the air but also in hyphae contacting hydrophobic solids (Claessen *et al*., 2002) under oxygen-limited conditions (van Keulen *et al*., 2003). This suggests that regulation of these *rdl* genes, and possibly other developmentally regulated genes, is not signalled through oxygen levels for instance. We propose that a molecule(s) accumulates in the cell wall of aerial hyphae, or hyphae in contact with a hydrophobic solid, that would otherwise diffuse into the medium. Accumulation of this molecule would be sensed and trigger rodlin expression. This mechanism would be similar to that proposed for pheromones in filamentous fungi, the so-called autocrine response (Hartmann *et al*., 1996; 1999).

Experimental procedures

Strains and plasmids

The following streptomycete strains were used: *S. coelicolor* M145 (Kieser *et al*., 2000), *S. coelicolor* Δ*rdlAB6* (Claessen *et al*., 2002), *S. coelicolor* Δ*chpABCDEH* (Claessen *et al*., 2003), *S. tendae* Tü901/8c (Richter *et al*., 1998), *S. griseus* (ATCC 13273), *S. avermitilis* (ATCC 31267) and *S. scabies* ISP5078. Cloning was done in *E. coli* DH5α, SCS110 or BW25113 (Datsenko and Wanner, 2000). *E. coli* ET12567 containing pUZ8002 was used for conjugation to *S. coelicolor* (Kieser *et al*., 2000). Vectors and constructs are summarized in Table 1.

Table 1. Vectors and constructs used in this work.

Plasmid	Description	Reference
pBluescript-II KS+	pUC18 derivative for cloning in *E. coli*	Stratagene
C61A	Cosmid 61A of *S. coelicolor* containing *chpF* and *chpG*	Redenbach *et al*. (1996)
pIJ82	pSET152 (Bierman *et al*., 1992) derivative containing the *hyg* gene, replacing a 751 bp *Sac*I fragment containing the *aac(3)IV* apramycin gene	Dr B. Gust (JIC)
pIJ82-*rdlA*	pIJ82 containing a 1501 bp fragment encompassing the putative promoter and coding sequence of *rdlA* as well as a 843 bp sequence 3′ of the stop codon of *rdlA*	This work
pIJ82-*rdlB*	pIJ82 containing a 1412 bp fragment encompassing the putative promoter and coding sequence of *rdlB* as well as a 748 bp sequence 3′ of the stop codon of *rdlB*	This work
pIJ8630*a*	pIJ8630 containing the 262 bp *S. coelicolor* promoter region of *rdlA* with an *Nde*I site at the 3′ end allowing translational fusions	Claessen *et al*. (2002)
pIJ8630*b*	As pIJ8630*a* but with the promoter region of *rdlB*	Claessen *et al*. (2002)
StC18	pBluescript-II KS+ derivative with a 3.1 kb *Sal*I fragment of cosmid C18 of *S. tendae* containing *STrdlA* and *STrdlB*	This work
StC60	pBluescript-II KS+ derivative with a 4.2 kb *Sal*I fragment of cosmid C60 of *S. tendae* containing *STrdlA* and *STrdlB*	This work
Sg12A10	pBluescript-II KS+ derivative with a 3.6 kb *Sal*I fragment of cosmid 12A10 of *S. griseus* containing *SGrdlA* and *SGrdlB*	This work
pIJ8630-StC18	pIJ8630 derivative (Sun *et al*., 1999) with a 3.2 kb *Kpn*I–*Xba*I fragment containing *STrdlA* and *STrdlB*	This work
pSET-Sg12A10	pSET152 derivative (Bierman *et al*., 1992) with a 2.6 kb *Bam*HI fragment of Sg12A10 containing *SGrdlA* and *SGrdlB*	This work

Growth conditions and media

Streptomyces strains were grown at 30°C on solid MS agar medium or in YEME medium as liquid shaken cultures (Kieser *et al*., 2000). R5 was used for regenerating protoplasts (Kieser *et al*., 2000). *S. griseus* was grown in liquid mNMMP (van Keulen *et al*., 2003) to obtain submerged spores.

For GFP studies, *Streptomyces* strains were grown on solid mNMMP medium (van Keulen *et al*., 2003).

Molecular techniques

Standard molecular techniques followed the methods described by Sambrook *et al*. (1989). Protoplast preparation and transformation were performed as described by Kieser *et al*. (2000). Chromosomal DNA was isolated according to the method of Verhasselt *et al*. (1989) and modified by the method of Nagy *et al*. (1995).

Total RNA of *S. coelicolor* was isolated according to the method of Veenendaal and Wösten (1998) or van Keulen *et al*. (2004). DNA and RNA were blotted on Nylon filters (Boehringer Mannheim) and hybridized under conditions described by Church and Gilbert (1984) at 62°C. Under these conditions, *rdlA* and *rdlB* do not cross-hybridize (Claessen *et al*., 2002).

For preparation of the *rdlA* and *rdlB* probes, the respective coding sequences were amplified using PCR with primers *rdlA*CSFW and *rdlA*CSREV for *rdlA*, and *rdlB*CSFW and *rdlB*CSREV for *rdlB* (*Supplementary material*, Table S1). The PCR products were radioactively labelled using the Prime-a-Gene® kit (Promega). For the *SGrdlA* probe, a 618 bp *Bst*XI–*Eco*RV fragment of plasmid Sg12A10 was labelled.

Isolation of rdl *homologues from* S. tendae *and* S. griseus

Coding sequences of *rdlA* and *rdlB* from *S. coelicolor* were radioactively labelled and hybridized to cosmid libraries of *S. tendae* Tü901/8c (Bormann *et al*., 1996) and *S. griseus* (Menéndez *et al*., 2004). Hybridizing *Sal*I fragments of positive clones were cloned in pBluescript-II KS+ and sequenced. Accession numbers for *SGrdlA*, *SGrdlB*, *STrdlA* and *STrdlB* are AJ630587, AJ630588, AJ630589 and AJ630590 respectively.

*Construction of M145Δ*rdlA*, M145Δ*rdlB*, M145Δ*rdlAB*, M145Δ*chpFG *and M145Δ*chpABCDEFGH

The Redirect© technology (Gust *et al*., 2003) was used to disrupt *rdlA* and/or *rdlB* of *S. coelicolor* M145. For the disruption of *rdlA*, the *aac(3)IV* resistance cassette was amplified using primers *rdlA*sense and *rdlA*ansense (*Supplementary material*, Table S1). Similarly, for the disruption of *rdlB*, primers *rdlB*sense and *rdlB*ansense were used. Primers *rdlA*ansense and *rdlB*ansense were used for the disruption of both *rdlA* and *rdlB*.

For the construction of the M145Δ*chpABCDEFGH* strain, lacking all *chp* genes, the apramycin cassette was removed in the Δ*chpABCDEH* strain enabling the reuse of this cassette to delete *chpF* and *chpG*. Primers used are shown in *Supplementary material*, Table S1. *chpF* and *chpG* were mutated on cosmid C61A (Redenbach *et al*., 1996) using the apramycin cassette. The cosmid containing the mutated copies of both genes was introduced into the Δ*chpABCDEH* strain, followed by screening for loss of both *chpF* and *chpG*. Similarly, *chpF* and *chpG* were deleted in the wild-type strain. Gene deletions were confirmed by Southern analysis.

Electron microscopy

For freeze-fracturing and cryoscanning electron microscopy, spores were frozen in a mixture of solid and liquid nitrogen. Freeze-fracturing was done in a Polaron freeze-etch apparatus equipped with a Balzers EVM 052 unit. Replicas of Pt/C were cleaned for 16 h in 40% chromic acid at room temperature. Cryoscanning electron microscopy was done with a Jeol microscope type 6301F at 5.0 kV using sputter coating with gold/palladium.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4143/mmi4143sm.htm

Fig. S1. Alignment of the amino acid sequences of the rodlins.

Table S1. Primers used in this study.

References

Bentley, S.D., Chater, K.F., Cerdeño-Tárraga, Challis, G.L., Thomson, N.R., James, K.D., *et al.* (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417:** 141–147.

Bibb, M.J., Molle, V., and Buttner, M.J. (2000) σ^{BldN}, an extracytoplasmic function RNA polymerase σ factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2). *J Bacteriol* **182:** 4606–4616.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schoner, B.E. (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116:** 43–49.

Bormann, C., Möhrle, V., and Bruntner, C. (1996) Cloning and heterologous expression of the entire set of structural genes for nikkomycin synthesis from *Streptomyces tendae* Tü901 in *Streptomyces lividans*. *J Bacteriol* **178:** 1216–1218.

Chater, K.F. (1998) Taking a genetic scalpel to the *Streptomyces* colony. *Microbiology* **144:** 1465–1478.

Chater, K.F. (2001) Regulation of sporulation in *Streptomyces coelicolor* A3(2): a checkpoint multiplex? *Curr Opin Microbiol* **4:** 667–673.

Church, G.M., and Gilbert, W. (1984) Genomic sequencing. *Proc Natl Acad Sci USA* **81:** 1991–1995.

Claessen, D., Wösten, H.A.B., van Keulen, G., Faber, O.G., Alves, A.M.C.R., Meijer, W.G., and Dijkhuizen, L. (2002) Two novel homologous proteins of *Streptomyces coelicolor and Streptomyces lividans* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface. *Mol Microbiol* **44:** 1483–1492.

Claessen, D., Rink, R., de Jong, W., Siebring, J., de Vreugd, P., Boersma, F.G.H., *et al.* (2003) A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. *Genes Dev* **17:** 1714–1726.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Elliot, M.A., Karoonuthaisiri, N., Huang, J., Bibb, M.J., Cohen, S.N., Kao, C.M., and Buttner, M.J. (2003) The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor*. *Genes Dev* **17:** 1727–1740.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003) Gene replacement by PCR targeting in *Streptomyces* and its use to identify a protein domain involved in the biosynthesis of the sesquiterpene odour geosmin. *Proc Natl Acad Sci USA* **100:** 1541–1546.

Hartmann, H.A., Kahmann, R., and Bölker, M. (1996) The pheromone response factor coordinates filamentous growth and pathogenicity in *Ustilago maydis*. *EMBO J* **15:** 1632–1641.

Hartmann, H.A., Kruger, J., Lottspeich, F., and Kahmann, R. (1999) Environmental signals controlling sexual development of the corn smut fungus *Ustilago maydis* through the transcriptional regulator Prf1. *Plant Cell* **11:** 1293–1306.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., *et al.* (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. *Nature Biotechnol* **21:** 526–531.

Kelemen, G.H., and Buttner, M.J. (1998) Initiation of aerial mycelium formation in *Streptomyces*. *Curr Opin Microbiol* **1:** 656–662.

Kendrick, K.E., and Ensign, J.C. (1983) Sporulation of *S. griseus* in submerged culture. *J Bacteriol* **155:** 357–366.

van Keulen, G., Jonkers, H.M., Claessen, D., Dijkhuizen, L., and Wösten, H.A.B. (2003) Differentiation and anaerobiosis in standing liquid cultures of *Streptomyces coelicolor*. *J Bacteriol* **185:** 1455–1458.

van Keulen, G., Siebring, J., Rembacz, K.P., Hoogeveen, M., Tomczynska, M., and Dijkhuizen, L. (2004) Improved method for the isolation of RNA from (standing liquid cultures of) streptomycetes. *J Microbiol Methods* (in press).

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., and Hopwood, D.A. (2000) *Practical Streptomyces Genetics*. Norwich: The John Innes Foundation.

Menéndez, N., Nur-E-Alam, M., Braña, A.F., Rohr, J., Salas, J.A., and Méndez, C. (2004) Biosynthesis of the antitumor chromomycin A3 in *Streptomyces griseus*: analysis of the gene cluster and rational design of novel chromomycin analogues. *Chem Biol* **11:** 21–32.

Nagy, I., Schoofs, G., Compernolle, F., Proost, P., Vanderleyden, J., and de Mot, R. (1995) Degradation of the thiocarbonate herbicide EPTC (S-ethyl dipropylcarbamotioate) and biosafening by *Rhodococcus* sp. strain NI86/21 involve an inducible cytochrome P-450 system and aldehyde dehydrogenase. *J Bacteriol* **177:** 676–687.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H., and Hopwood, D.A. (1996) A set of ordered cosmids and a detailed genetic and physical map of the 8 Mb *Streptomyces coelicolor* A3(2) chromosome. *Mol Microbiol* **21:** 77–96.

Richter, M., Willey, J.M., Süßmuth, R., Jung, G., and Fiedler, H.-P. (1998) Streptofactin, a novel biosurfactant with aerial mycelium inducing activity from *Streptomyces tendae* Tü 901/8c. *FEMS Microbiol Lett* **163:** 165–171.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Smucker, R.A., and Pfister, R.M. (1978) Characteristics of *Streptomyces coelicolor* A3(2) aerial spore rodlet mosaic. *Can J Microbiol* **24:** 397–408.

Sun, J., Kelemen, G.H., Fernández-Abalos, J.M., and Bibb, M.J. (1999) Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *J Bacteriol* **145:** 2221–2227.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22:** 4673–4680.

Tillotson, R.D., Wösten, H.A.B., Richter, M., and Willey, J.M. (1998) A surface active protein involved in aerial hyphae formation in the filamentous fungus *Schizophyllum commune* restores the capacity of a bald mutant of the filamentous bacterium *Streptomyces coelicolor* to erect aerial structures. *Mol Microbiol* **30:** 595–602.

Veenendaal, A.K.J., and Wösten, H.A.B. (1998) SV total RNA isolation system in action. Total RNA from the Gram-positive bacterium *Streptomyces lividans*. *Promega Benelux News* **17:** 7.

Verhasselt, P., Poncelet, F., Vits, K., and Vanderleyden, J. (1989) Cloning and expression of a *Clostridium acetobutylicum* alpha-amylase gene in *Escherichia coli*. *FEMS Microbiol Lett* **50:** 135–140.

Wildermuth, H., Wehrli, E., and Horne, R.W. (1971) The surface structure of spores and aerial mycelium in *Streptomyces coelicolor*. *J Ultrastruct Res* **35:** 168–180.

Willey, J.M., Santamaria, R., Guijarro, J., Geistlich, M., and Losick, R. (1991) Extracellular complementation of a developmental mutation implicates a small sporulation protein in aerial mycelium formation by *S. coelicolor*. *Cell* **65:** 641–650.

Willey, J.M., Schwedock, J., and Losick, R. (1993) Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Dev* **7:** 895–903.

Wösten, H.A.B., and Willey, J.M. (2000) Surface-active proteins enable microbial aerial hyphae to grow into the air. *Microbiology* **146:** 767–773.

Wösten, H.A.B., de Vries, O.M.H., and Wessels, J.G.H. (1993) Interfacial self-assembly of a fungal hydrophobin into a hydrophobin rodlet layer. *Plant Cell* **5:** 1567–1574.